Neuron, Vol. 40, 871–873, December 4, 2003, Copyright ©2003 by Cell Press

Finding Your Way to the End: A Tale of GDNF and Endothelin-3

Both RET and EDNRB are essential for neural crest development in many parts of the embryo. In this issue of *Neuron*, two articles by Barlow et al. and Kruger et al. demonstrate that within the developing enteric nervous system these receptors have synergistic, stage-dependent effects on neural crest precursor proliferation and antagonistic effects on cell migration.

Neural crest cells originate in the dorsal neural tube and migrate extensively through the developing embryo. During their migration they receive signals from the environment that control their migratory path, regulate proliferation, restrict their developmental potential, determine their viability, and encourage the formation of differentiated cells. Neural crest derivatives form a broad array of cell types including neurons and glia of the peripheral sensory, autonomic, and enteric nervous system, melanocytes, adrenal medullary cells, cardiac great vessels, and parts of the cranial musculoskeletal system. As these cells migrate and differentiate, they change both their cell surface receptors and their intracellular signal transduction pathways to alter their response to environmental signals. Two papers in this issue of *Neuron* by Kruger et al. (2003) and Barlow et al. (2003) demonstrate that closely related neural crest cell populations destined to form the enteric nervous system may have different responses to the same trophic factors even though they express the same cell surface receptors.

The enteric nervous system (ENS) is a complex network of neurons and glia within the wall of the gut that controls intestinal motility, responds to sensory stimuli from the gut lumen, and regulates intestinal secretion and blood flow. To perform these functions, there are at least 14 different types of enteric neurons. All of these cells originate in the vagal and sacral neural crest. Colonization of the gut by these neural crest precursors requires coordinated cell migration, proliferation, and differentiation that is regulated by specific molecular signals both within the neural crest and in the intestinal environment (Gariepy, 2001). Although a wide variety of problems may occur during ENS development, the best-understood abnormality is called Hirschsprung's disease (HSCR). This disorder, which affects 1:5000 human infants, is a developmental anomaly that results in the complete absence of enteric neurons and glia in the distal bowel. Distal intestinal aganglionosis results in tonic contraction of the aganglionic bowel that may be fatal without surgical treatment.

The genetics of Hirschsprung's disease is complex, with mutations in eight different genes found in affected individuals (Carrasquillo et al., 2002). HSCR genetics is further complicated by incomplete penetrance and phenotypic variability within and between families, suggesting that genetic interactions, environmental exposures, or stochastic developmental processes influence the HSCR phenotype. Mutations in two specific genes, *Ret* and endothelin receptor B (EDNRB), are the most common genetic causes for HSCR in humans, accounting for up to 50% and 5% of HSCR, respectively. Many of the other molecules implicated in HSCR also modulate the activity of these receptors.

Ret is a transmembrane tyrosine kinase receptor that responds to four different ligands (glial cell line-derived neurotrophic factor [GDNF], neurturin [NRTN], artemin [ARTN], and persephin [PSPN]) (Baloh et al., 2000). Because *Ret* does not bind to these ligands directly, the responsiveness of *Ret*-expressing cells to particular ligands is dependent on the expression of the *Ret* coreceptors GFRα1, GFRα2, GFRα3, or GFRα4. Each coreceptor preferentially binds to a specific ligand to form functional interactions in vitro and in vivo (GFRα1/GDNF, GFRα2/NRTN, GFRα3/ARTN, or GFRα4/PSPN). Activation of *Ret* stimulates multiple intracellular signal transduction pathways (MAPK, PI-3 kinase/Akt, Src, PLC-γ), and recent evidence suggests that GDNF may mediate *Ret*-independent signaling via neural cell adhesion molecule (NCAM). *Ret* activation supports enteric neural crest survival, proliferation, migration, and axon extension. Activation of *Ret* is critical for formation of the ENS, as demonstrated by the absence of enteric neurons distal to the stomach in $Ret^{-/-}$, GFRα$1^{-/-}$, or $GDNF^{-/-}$ mice. EDNRB is a G protein-coupled 7-transmembrane-spanning receptor that stimulates phosphatidylinositol phosphate turnover, increases intracellular Ca^{2+}, and activates MAPK via $G\alpha_q$; enhances arachidonic acid release via phospholipase A2; and reduces cAMP production through $G\alpha_i$. EDNRB can be activated by three ligands (endothelin-1 [ET-1, also known as EDN1], endothelin-2 [ET-2, also known as EDN2], and endothelin-3 [ET-3, also known as EDN3]), but only the interaction with ET-3 is essential for formation of the ENS. (For the sake of convenience and consistency, I will be referring to these ligands as ET-1, -2, and -3.) $ET\text{-}3^{-/-}$ and $EDNRB^{-/-}$ mice have distal colonic aganglionosis, suggesting that EDNRB signaling is critical for colonization of the colon by ENS precursors (Baynash et al., 1994; Puffenberger et al., 1994). There is still limited knowledge about which signal transduction pathways are essential for which aspects of neural crest development and even less is known about how neural crest cells integrate multiple extracellular signals to follow a specific cell fate decision.

Genetic interactions between *Ret* and EDNRB have demonstrated that these signaling systems act synergistically in both mice (McCallion et al., 2003) and humans (Carrasquillo et al., 2002) to allow colonization of the distal bowel. The articles by Barlow et al. and Kruger et al. in this issue of *Neuron* expand on these observations to demonstrate that in addition to the effects of ET-3 in the colon, endothelin signaling is critical for formation of the ENS within the small bowel. EDNRB-defi-

cient rats have fewer neural crest stem cells in the small bowel than wild-type rats (Kruger et al., 2003). Mice missing endothelin-3 (ls/ls) also have hypoganglionosis in the small bowel that is dramatically exacerbated by replacing wild-type *Ret* with a weakened form of the *Ret* receptor (*Ret*^{51}/*Ret*^{51}). Curiously, replacing only a single *Ret* allele with *Ret*^{51} (i.e., *Ret*$^{+}$/*Ret*^{51}) results in a partial rescue of the ls/ls phenotype (compared to ls/ls; Ret^{+}/Ret^{+}), suggesting that there are complex interactions between the *Ret* and EDNRB signaling pathways that are essential for formation of the ENS (Barlow et al., 2003).

The precise biological role for EDNRB signaling in ENS development has been difficult to determine. Because treatment of isolated mouse intestine with an EDNRB inhibitor prevents colonization of the distal bowel at E12.5, EDNRB activity must be required by cells within the bowel rather than by premigratory neural crest precursors that also express EDNRB (Woodward et al., 2000). Identifying the cells within the bowel that depend on EDNRB has been more challenging, since EDNRB is expressed both in the migrating neural crest cells and in the gut mesenchyme. Indeed, experiments with aggregation chimeras demonstrated that EDNRB-deficient neural crest cells can migrate into the distal bowel if there are enough wild-type cells within the colon (Kapur et al., 1995). This suggests that EDNRB signaling may modify the gut microenvironment to allow neural crest migration into the distal bowel. Interestingly, ET-3 deficiency results in increased laminin production in the colon. Because laminin promotes neuronal differentiation, it has been proposed that ET-3 deficiency results in premature differentiation of neural crest cells along a neuronal lineage and that this premature differentiation inhibits the migration of these cells into the distal bowel (Gershon, 1995).

From these experiments arose some straightforward hypotheses that have been tested and in some cases refuted by the Morrison's and Pachnis's groups in this issue of *Neuron*. First is the hypothesis that ET-3 prevents premature neuronal differentiation of neural crest cells within the bowel and encourages continued proliferation of neural crest precursors. While this may appear to be a simple hypothesis, the results are complicated by the fact that the enteric neural crest is an evolving cell population that changes its response to trophic factors as it undergoes lineage restriction. Morrison's study (Kruger et al., 2003) focuses on p75$^{+}\alpha_4$ integrin^{+} enteric neural crest stem cells (NCSC) that his group has previously demonstrated have a remarkable capability for proliferation as well as the ability to form several differentiated cell types and to self-renew (i.e., form additional multipotent daughter cells) in culture. Using a variety of different approaches, they demonstrate that EDNRB is not required for NCSC proliferation or self-renewal in culture and that EDNRB deficiency does not affect the cell cycle distribution of NCSC freshly isolated from the small bowel. Furthermore, EDNRB deficiency did not lead to progressive loss of NCSC after E12.5 and did not cause increased neuronal differentiation or cell death in the ileum or cecum in vivo. Finally, ET-3 did not strongly affect the ability of NCSC to differentiate into neurons in the presence of BMP4. Interestingly, added ET-3 did impair self-renewal of NCSC in vitro and markedly reduced the number of neuron-containing colonies in the absence of BMP4. The fact the most NCSC differentiated into smooth muscle actin-expressing myofibroblasts in BMP4-deficient/ET-3-treated cultures is interesting, but because neural crest cells do not give rise to myofibroblasts in vivo, this differentiation pathway must be blocked by other factors in the intestinal microenvironment. Thus, there is not clear evidence that ET-3 prevents premature differentiation of NCSC into neurons or is required for NCSC proliferation.

The absence of an effect of EDNRB deficiency on NCSC proliferation is striking, given the ability of ET-3 to promote proliferation of neural crest cells as they exit from the neural tube. It also contrasts with the synergistic effect of ET-3 and GDNF on the proliferation of Ret^{+}/TuJ1^{-} enteric neural crest precursors in culture and the reduction in proliferation of SOX10-expressing cells in ET-3-deficient mouse embryos (Barlow et al., 2003). All of these results highlight the fact that there are many distinct stages of neural crest differentiation within the bowel wall. It suggests that rat p75$^{+}\alpha_4$ integrin^{+} NCSC are not comparable to mouse Ret^{+}/TuJ1^{-} or SOX10^{+} ENS precursors despite the fact that these are all immature enteric neural crest cells. This is further highlighted by the observation that although NCSC express *Ret* and GFRα1, they do not proliferate in response to GDNF at least under the conditions used to grow these cells in culture (Iwashita et al., 2003). This is in marked contrast to the strong proliferative effect of GDNF on most *Ret*-expressing enteric neural crest cells in vitro and in vivo. In addition, the observation that EDNRB-deficient rats have a marked reduction in NCSC and that ET-3-deficient mice have a reduced number of Phox2b-expressing neural crest precursor cells in the small bowel demonstrates that the effect of ET-3 is not limited to the colon and implies that ET-3 affects the survival, proliferation, migration, or differentiation of NCSC before E12.5. It will be interesting to determine why NCSC and other immature enteric neural crest derivatives respond so differently to GDNF and ET-3 both in vitro and in vivo. It suggests that these NCSC have additional signaling pathways activated that modify their response to GDNF and ET-3.

The second critical hypothesis addressed by both Barlow et al. and Kruger et al. is that ET-3 enhances the ability of neural crest cells to migrate into the distal bowel. Although the simplest way for ET-3 to do this would be to enhance the rate of neural crest migration, EDNRB-deficient neural crest cells migrate out of the bowel normally in response to GDNF (Kruger et al., 2003). In addition, both studies demonstrate that ET-3 dramatically reduces the migration of neural crest cells from the bowel and toward a source of GDNF. This surprising finding might explain some previous observations that have been confusing. For example, there is a dramatic increase in the number of myenteric neurons in the colon compared to the small bowel in wild-type mice. One way to generate this increased neuron number is to provide the enteric neural crest precursors with a stronger mitogenic signal as they enter the colon. Thus, during embryogenesis, when neural crest cells enter the colon, the highest levels of both ET-3 and GDNF are found in the cecum. These factors should cooperatively increase the proliferation of non-NCSC

$Ret^+/TuJ1^-$ ENS precursors within the colon. Increasing the concentration of GDNF in the cecum, however, creates a new problem. If the mitogenic signal is also the primary chemoattractive signal toward which these cells migrate, then why should ENS precursors migrate beyond the cecum? One explanation is that as these cells proliferate in the cecum, they compete for the available GDNF and then eventually migrate toward areas of the distal bowel where there are fewer *Ret*-expressing cells and more free GDNF. While this may be part of the mechanism, these articles suggest that the high level of ET-3 in the cecum also reduces the migration of neural crest cells toward GDNF sources and allows these cells to migrate toward another, as yet undefined, trophic factor.

While the manuscripts by Morrison's and Pachnis's research groups each individually provide new insight into the role of endothelin receptor B signaling in ENS development, the contrasting results in these manuscripts demonstrate how complex the cell fate decisions are that allow neural crest cells to populate the bowel. The differences in response to GDNF and ET-3 between NCSC and other early enteric neural crest derivatives are particularly striking. The ability of NCSC to proliferate in culture in the absence of *Ret* (Iwashita et al., 2003) or EDNRB (Kruger et al., 2003) signaling raises the possibility that these cells could be used to repopulate the distal aganglionic bowel in patients with Hirschsprung's disease. This would then allow autologous transplants of NCSC to the distal bowel, eliminating the need for immunosuppression. The ability of these mutant cells to form mature neurons or to establish an interacting functional network of ganglion cells within the distal bowel remains to be established.

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Synaptic Inhibition Mediated by Glia

It has become increasingly clear over the past several years that astrocytes can release chemical transmitters in response to the activation of neurotransmitter receptors. In this issue of *Neuron*, Zhang et al. demonstrate that the regulated release of one of these gliotransmitters, ATP, mediates a form of heterosynaptic suppression.

Research over the last decade has changed our view of the integrative capacity of astrocytes, a subtype of glial cell. Through groundbreaking work from several laboratories, we now know that these cells are much more than "glue"; they play essential roles in nervous system development and function (Volterra et al., 2002). Some surprising observations have been made during this time. Astrocytes express a plethora of neurotransmitter receptors that can induce calcium signaling, which in turn leads to the release of chemical transmitters. But what are the roles of these gliotransmitters? In this issue of *Neuron*, Zhang et al. (2003) detail their elegant experiments that demonstrate that one of these gliotransmitters, ATP, regulates synaptic transmission and causes heterosynaptic modulation between hippocampal neurons.

It is known that in area CA1 of the hippocampus, two-thirds of the axon-dendritic spines are associated with a process of an astrocyte (Ventura and Harris, 1999). Much attention has been focused on this tripartite synapse (Araque et al., 1999) for the uptake of chemical transmitters released at the synapse as well as for the regulation of energy transfer from the vasculature through the interposed astrocyte to the synapse (Nedergaard et al., 2003). Zhang et al.'s study adds to our understanding both by demonstrating that ATP released from astrocytes leads to a depression of synaptic transmission and by showing that heterosynaptic suppresion is modulated by the interposed astrocytes.

Strengthening these studies is the fact that they were performed both in cell culture, where synapses are more readily experimentally accessible, and then in hippocampal slices. Initially, using mixed cultures of neurons and astrocytes, these researchers show that pharmacological antagonists of metabotropic purinergic receptors, P2Y receptors, augment synaptic transmission. Exogenous addition of ATP depresses the connection, an action that is blocked by P2Y receptor antagonists, suggesting that there is a tonic release of ATP that depresses synaptic transmission. Modulation of these cultured synapses is judged to be presynaptic based on a variety of approaches, including a reduction in somatic calcium current, a decrease in mEPSC frequency, and a reduced stimulus-dependent depression of the con-